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Formation and Persistence of Arylamine DNA Adducts *In Vivo*

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Aromatic amines are urinary bladder carcinogens in man and induce tumors at a number of sites in experimental animals including the liver, mammary gland, intestine, and bladder. In this review, the particular pathways involved in the metabolic activation of aromatic amines are considered as well as the specific DNA adducts formed in target and nontarget tissue. Particular emphasis is placed on the following compounds: 1-naphthylamine, 2-naphthylamine, 4-aminobiphenyl, 4-acetylaminobiphenyl, 4-acetylamino-4'-fluorobiphenyl, 3,2'-dimethyl-4-aminobiphenyl, 2-acetylaminofluorene, benzidine, *N*-methyl-4-aminoazobenzene, 4-aminoazobenzene, and 2-acetylaminophenanthrene.

Introduction

Aromatic amines and amides were originally associated with the induction of bladder tumors in man and have subsequently been found to be carcinogenic at a number of sites in a variety of experimental animals (*1,2*). The initial step in the metabolic activation of these compounds usually involves an *N*-oxidation (Fig. 1), which can be catalyzed by cytochrome P-450, the flavin-containing monooxygenase (FMO), or peroxidases, such as prostaglandin H synthase (PHS). Primary arylamines are *N*-oxidized to *N*-hydroxy arylamines that can undergo an acid-catalyzed reaction with DNA or can be further metabolized to a reactive electrophile through acetyl coenzyme A (AcCoA)-dependent *O*-acetylation, ATP-dependent *O*-aminoacylation, or 3'-phosphoadenosine 5'-phosphosulfate (PAPS)-dependent *O*-sulfonylation. The *N*-oxidation of secondary arylamines results in *N*-hydroxy metabolites that do not react directly with DNA but are further converted into electrophilic derivatives by *O*-sulfonylation.

Aromatic amides are *N*-oxidized only by the cytochrome P-450 monooxygenases, and since the *N*-hydroxy arylamides (arylhydroxamic acids) that are formed

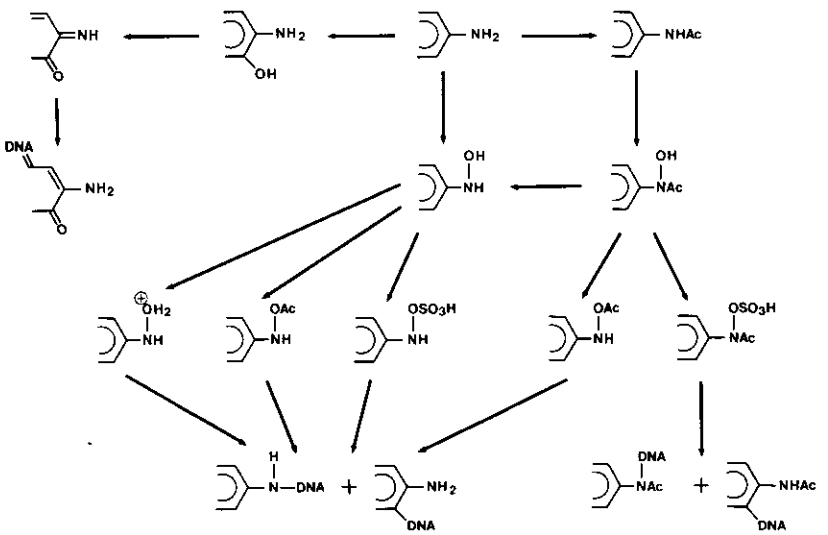

FIGURE 1. Metabolic pathways leading to DNA adducts from arylamines and arylamides.

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are not reactive, further metabolic activation is required. This can be accomplished by at least three pathways: *O*-sulfonylation, deacetylation to a *N*-hydroxy arylamine, or intramolecular *N,O*-acyltransfer to a *N*-acetoxy arylamine. In this review, we consider how these various metabolic pathways lead to the specific arylamine and arylamide DNA adducts that have been detected *in vivo*. We also discuss the relationship between the persistence of specific adducts and tumor induction.

1-Naphthylamine

Although 1-naphthylamine (1-NA) has not been found to be carcinogenic in any bioassay (*1,3,4*), its *N*-oxidized derivative, *N*-hydroxy-1-NA, is strongly carcinogenic at sites of application (*5–7*). The *in vitro* reaction of *N*-hydroxy-1-NA with DNA yields at least three adducts (Fig. 2): *N*-(deoxyguanosin-O^6-yl)-1-NA, 2-(deoxyguanosin-O^6-yl)-1-NA, and a decomposition product of the latter adduct (*8*). *N*-(Guan-8-yl)-1-NA has also been isolated after acidic hydrolyis of *N*-hydroxy-1-NA-treated DNA (*9*) but has not been detected after enzymatic hydrolysis (Kadlubar, unpublished observation). As with other primary *N*-hydroxy arylamines, DNA adduct formation with *N*-hydroxy-1-NA is acid-catalyzed and is 20-fold greater at pH 5 as compared to pH 7.

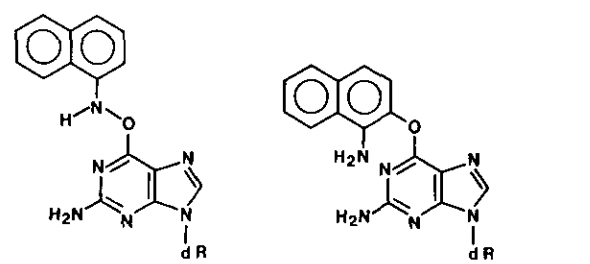

FIGURE 2. DNA adducts derived from 1-naphthylamine.

The DNA adducts formed at the subcutaneous injection site of rats administered *N*-hydroxy-1-NA have recently been examined (*7*). *N*-(Deoxyguanosin-O^6-yl)-1-NA was the major adduct detected 1 day after treatment and the total binding decreased by only 30% during the next 6 days. *N*-Hydroxy-1-NA bound to the DNA 20-fold better than the isomeric *N*-hydroxy-2-NA, which was consistent with the relative carcinogenicity of the two compounds. Furthermore, alternating the administration of both *N*-hydroxy arylamines decreased both the incidence of *N*-hydroxy-1-NA-induced tumors and the persistence of the adducts resulting from this compound.

Dogs have been used extensively as a model for arylamine carcinogenesis in man. Therefore, as part of a study relating the extent of adduct formation to carcinogenic potency, the binding of 1-NA to hepatic and urothelial DNA of dogs was assessed (*10,11*). Adduct formation from 1-NA was not detected in urothelial DNA and only very low levels were found in hepatic DNA. The failure to detect significant binding could indicate very efficient DNA repair processes or, alternatively, may indicate that 1-NA cannot be activated *in vivo*. The latter possibility appears more likely because in a recent study (*12*), cytochromes P-450 and FMO from a number of species were unable to catalyze the oxidation of 1-NA to *N*-hydroxy-1-NA.

2-Naphthylamine

In contrast to 1-NA, its isomer 2-NA is carcinogenic in a number of species including rats, dogs, and humans (*1*). When the *N*-oxidized derivative, *N*-hydroxy-2-NA, was reacted *in vitro* at pH 5 with DNA, three adducts were detected (Fig. 3): an imidazole ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA(50%), 1-(deoxyguanosin-*N*^{2}-yl)-2-NA (30%)), and 1-(deoxyadenosin-*N*^{6}-yl)-2-NA (15%) (*13*). Although 2-NA is carcinogenic and 1-NA is not, it should be noted that the *N*-hydroxy derivative of 2-NA does not bind to DNA *in vitro* nearly as well as does *N*-hydroxy-1-NA (*14*). This difference in binding also occurs *in vivo* because, as mentioned above, *N*-hydroxy-2-NA bound to injection site DNA in rats to a much lower extent than was found with *N*-hydroxy-1-NA (*7*). This reduced binding was probably responsible for the small tumor yield being recorded with *N*-hydroxy-2-NA.

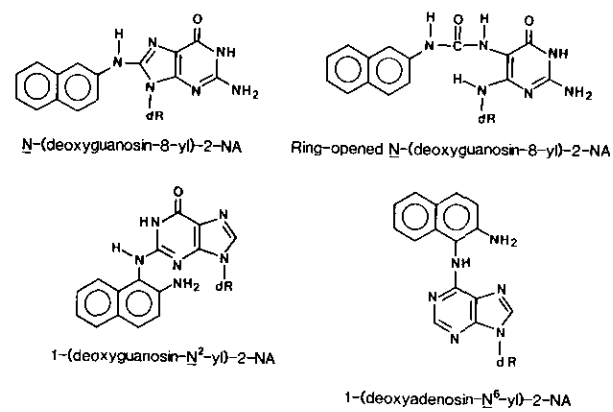

FIGURE 3. DNA adducts derived from 2-naphthylamine.

The DNA adduct derived from 2-NA in target (urothelium) and nontarget (liver) tissues of dogs have also been reported (*10,11*). Two days following the oral administration of 2-NA, the same three adducts that were found *in vitro* from *N*-hydroxy-2-NA were detected in both tissues with 4-fold higher binding levels being observed in the urothelial DNA. The major product in both tissues was the ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA, followed by lower levels of 1-(deoxyadenosin-*N*^{6}-yl)-2-NA and 1-(deoxyguanosin-*N*^{2}-yl)-2-NA. Although there is not an exact correspondence between the ratios of adducts found *in vivo* and *in vitro*, the commonality of adducts suggests that *N*-hy-

droxy-2-NA could be the ultimate carcinogenic species *in vivo*. This view is strengthened by a pharmacokinetic model which correctly predicts bladder tumor susceptibility in different species as a function of urine pH, voiding interval and resorption (*15*).

When the extent of binding was measured 7 days after 2-NA administration, the N^2-deoxyguanosine adduct appeared to persist in the dog liver, while both this adduct and the C8-deoxyguanosine adduct persisted in the bladder(*10,11*). The differential loss of adducts indicates that active repair processes are present in both tissues, and the relative persistence of the C8-deoxyguanosine adduct in target but not in the nontarget tissue suggests that this adduct may be a critical lesion for the initiation of urinary bladder tumors.

Peroxidative enzymes, such as PHS, have been shown to catalyze both the *N*- and ring-oxidation of 2-NA (*16*). When DNA was included in these *in vitro* incubations, six adducts were detected (*17*), three arising from *N*-hydroxy-2-NA, while the remaining three appeared to be formed from 2-imino-1-naphthoquinone, the oxidative product of 2-amino-1-naphthol (Fig. 4). Since PHS activity has been demonstrated in the dog bladder (*18*), additional experiments were conducted to determine if 2-amino-1-naphthol-derived adducts could be detected *in vivo* (*17*). Two days following the administration of 2-NA, adducts that accounted for about 20% of the total binding and that were indicative of 2-imino-1-naphthoquinone formation were identified in urothelial but not liver DNA. Thus, it appears that PHS may have a significant role in arylamine bladder carcinogenesis.

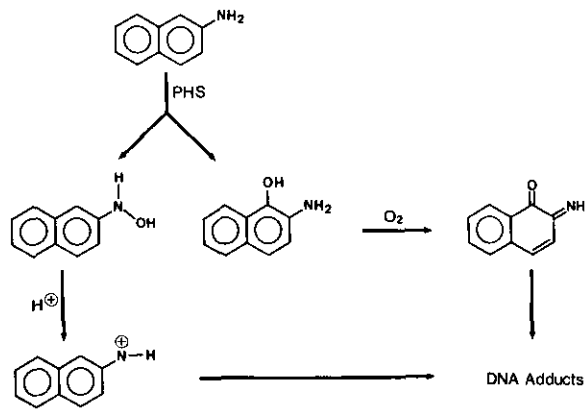

FIGURE 4. Metabolic activation of 2-naphthylamine by prostaglandin H synthase.

4-Aminobiphenyl and 4-Acetylaminobiphenyl

The arylamide, 4-acetylaminobiphenyl (AABP), differs from a number of aromatic amides in that its primary target in rats is the mammary gland as opposed to the liver (*19*). Although the adducts in the mammary gland have not been characterized, Kriek (*20*) has compared the binding of AABP to hepatic DNA with that of the strong hepatocarcinogen 2-acetylaminofluorene (AAF) in order to determine why AABP is such a poor inducer of liver tumors. Following administration of a single dose of the presumed proximate carcinogen, *N*-hydroxy-AABP, the total binding to hepatic DNA was about 25% of that found with the hydroxamic acid of AAF. When the individual adducts were examined, approximately 20% retained their acetyl groups and thus presumably arose from a sulfotransferase-catalyzed intermediate, while the remaining 80% were nonacetylated and may have resulted from deacetylation, *N,O*-acetyltransfer (*21*), or deacetylation followed by acetyl coenzyme A-dependent *O*-acetylation (*22*). Although the nonacetylated adducts were not characterized, the acetylated adducts were subsequently identified (*23*) as 3-(deoxyguanosin-N^2-yl)-AABP and *N*-(deoxyguanosin-8-yl)-AABP (Fig. 5).

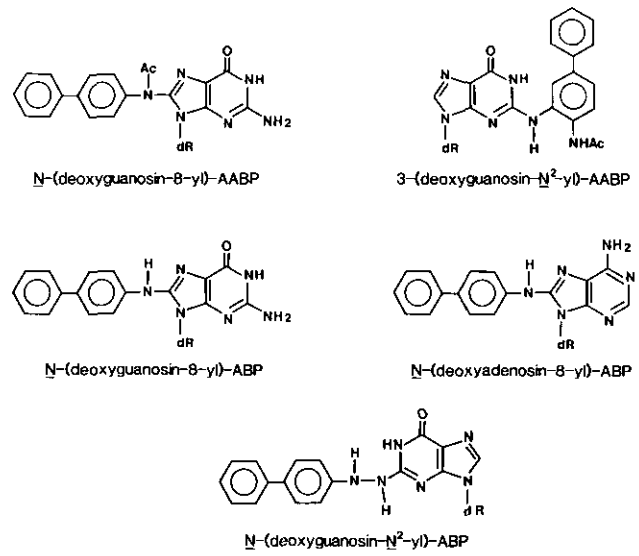

FIGURE 5. DNA adducts derived from 4-acetylaminobiphenyl and 4-aminobiphenyl.

Recently Gupta and Dighe (*24*) have used a ^{32}P-postlabeling technique to examine the types and persistence of adducts following the IP administration of several arylhydroxamic acids to rats. Maximum binding of *N*-hydroxy-AABP to hepatic DNA was observed 30 min after treatment but the value was only about 10% of the maximum value found with a similar dose of *N*-hydroxy-AAF. Initially, the binding rapidly decreased, by 50% in 4 hr and 75% in 24 hr, and then more slowly, resulting in approximately 10% of the adducts persisting after 29 days. The major adduct (ca. 70%) was a nonacetylated product which coeluted with a *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (ABP) standard (Fig. 5), while an additional nonacetylated adduct accounted for 7% of the initial binding. Two acetylated adducts were also detected; *N*-(deoxyguanosin-8-yl)-AABP was the major acetylated product (13%), while an adduct tentatively identified as 3-(deoxyguanosin-N^2-yl)-AABP was found in smaller amounts. After 29 days, the only adducts that were detected were the two nonacetylated ones as well as 3-(deoxyguanosin-N^2-yl)-AABP.

Among the aromatic amine carcinogens, ABP is the most potent bladder carcinogen in dogs (*25*). Since dogs are deficient in *N*-acetylases (*26,27*), *N*-hydroxy arylamines have been proposed to be the ultimate carcinogenic form in this species. When *N*-hydroxy-ABP was reacted with calf thymus DNA at pH 5, three adducts resulted (Fig. 5): *N*-(deoxyguanosin-8-yl)-ABP (80%), *N*-(deoxyadenosin-8-yl)-ABP (15%), and *N*-(deoxyguanosin-N^2-yl)ABP (5%) (*11,28*). The deoxyadenosine adduct was unusual in that it was substituted at C8, while the N^2-deoxyguanosine adduct was interesting because it contained a hydrazo linkage. When dogs were administered ABP a similar ratio of adducts (*11,28*) was found with *N*-(deoxyguanosin-8-yl)-ABP being the major product (76%), followed by *N*-(deoxyguanosin-N^2-yl)-ABP (15%), and then *N*-(deoxyadenosin-8-yl)-ABP (9%). Binding to urothelial DNA increased between 1 and 2 days, and appeared to remain constant for an additional 5 days. ABP gave the most extensive binding of the arylamine carcinogens examined, which correlated with it being the strongest carcinogen. However, essentially the same level of binding, adduct distribution and persistence were found in the liver, which is not a target for ABP.

4-Acetylamino-4′-fluorobiphenyl

Substitution of AABP with a 4′-fluoro group results in a compound that induces renal as well as hepatic and mammary gland tumors in rats (*29–31*). Kriek and Hengeveld (*32*) have examined the DNA adducts present in both liver and kidney in rats administered a single dose of the arylhydroxamic acid, *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl (AAFBP). A similar level of binding was found in both tissues with the majority (80%) of the adducts being nonacetylated. The nonacetylated adducts were not characterized but were suggested to be decomposition products of *N*-(deoxyguanosin-8-yl)-4-amino-4′-fluorobiphenyl (AFBP) (Fig. 6). Two acetylated adducts were found in equal concentrations; one was identified as *N*-(deoxyguanosin-8-yl)-AAFBP while the other was proposed to be 3-(deoxyguanosin-N^2-yl)-AAFBP. At nontoxic doses (5 mg/kg), the nonacetylated adducts were lost from both tissues with a half-life of 10 days; *N*-(deoxyguanosin-8-yl)-AAFBP was removed much faster ($t_{1/2}$ = 2 days), while 3-(deoxyguanosin-N^2-yl)-AAFBP appeared to be a persistent lesion. When a 25 mg/kg dose was administered, there was an initial fast rate of removal of all adducts in the kidney which was attributed to a toxic response.

3,2′-Dimethyl-4-aminobiphenyl

3,2′-Dimethyl-4-aminobiphenyl (DMABP) is unique among arylamines in that it is primarily a colon carcinogen in rats (*33–35*). In a manner analogous to arylamine bladder carcinogens, DMABP has been proposed (*36*) to be *N*-oxidized and *N*-glucuronidated in the liver to yield *N*-hydroxy-DMABP-*N*-glucuronide, which is transported in the bile to the intestine where it can be

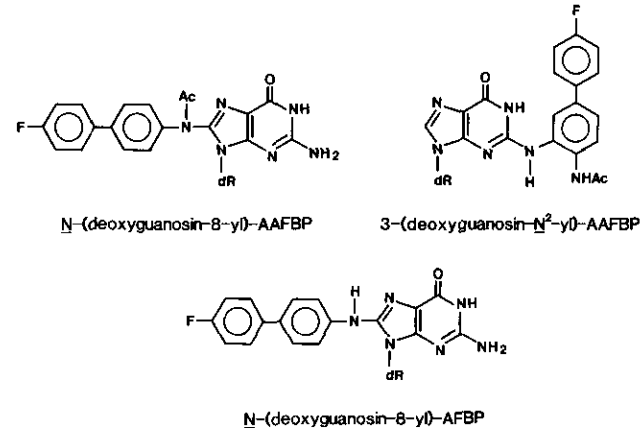

FIGURE 6. DNA adducts derived from 4-acetylamino-4′-fluorobiphenyl.

cleaved to release *N*-hydroxy-DMABP. Nussbaum et al. (*36*) have provided support for this mechanism by detecting *N*-hydroxy-DMABP-*N*-glucuronide as a biliary metabolite of DMABP. Flammang et al. (*37*) have shown that *N*-hydroxy-DMABP will react with DNA *in vitro* and that the extent of binding is greatly increased by AcCoA-dependent transacetylation that presumably yields *N*-acetoxy-DMABP. Further evidence for *N*-hydroxy-DMABP as a proximate carcinogen has been obtained by comparing hepatic and intestinal DNA adducts following a single subcutaneous injection of DMABP or its hydroxamic acid, *N*-acetyl-*N*-hydroxy-DMABP (*38*). In both tissues, two adducts were found: *N*-(deoxyguanosin-8-yl)-DMABP (80%) and 5-(deoxyguanosin-N^2-yl)-DMABP (20%) (Fig. 7). *N*-Acetylated adducts were not detected, and the extent of binding as well as the adduct profile obtained from *N*-acetyl-*N*-hydroxy-DMABP was nearly the same as that found with DMABP. These data suggest that *N*-hydroxy-DMABP serves as a common intermediate for both compounds.

The initial binding of DMABP to hepatic DNA was approximately twice that observed in intestinal DNA and the adducts in the liver were more persistent with only 30% being lost within 7 days as compared to 70% in the intestine. The greater persistence and higher adduct levels found in the liver appeared to be inconsistent with the tissue specificity of DMABP. However, when the rates of *de novo* DNA synthesis were measured, the rate in the intestine was at least 20 times that found in the liver. It seems, therefore, that although total binding is lower in the intestinal DNA, the highly

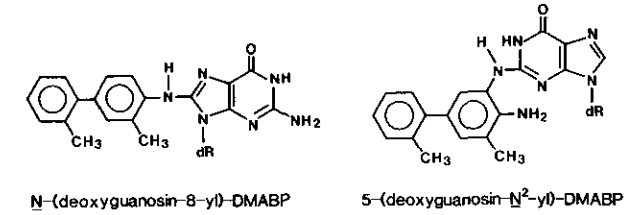

FIGURE 7. DNA adducts derived from 3,2′-dimethyl-4-aminobiphenyl.

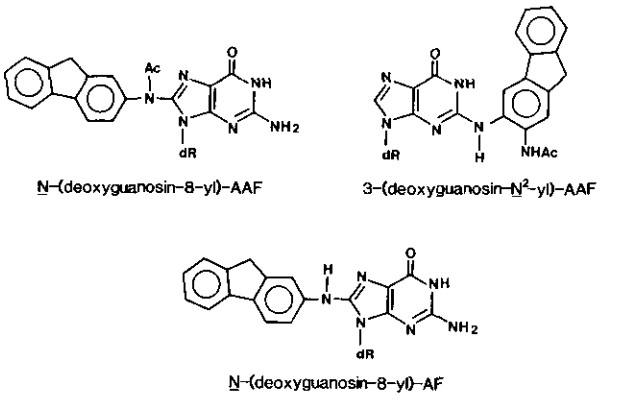

FIGURE 8. DNA adducts derived from 2-acetylaminofluorene.

proliferative nature of this tissue increases the probability that these adducts will serve as initiating lesions.

2-Acetylaminofluorene

2-Acetylaminofluorene (AAF), which is undoubtedly the most extensively studied of the arylamine carcinogens, induces tumors in a number of species and at a number of sites including liver, mammary gland, intestine, and urinary bladder (*39–41*). In rat liver, both acetylated and nonacetylated adducts are formed with the latter products accounting for approximately 80% of the total binding after a single administration of AAF or *N*-hydroxy-AAF (*42–44*). The major acetylated adduct in rat liver, which has been identified as *N*-(deoxyguanosin-8-yl)-AAF (Fig. 8), contributes about 15% of the total binding and has a half-life of 7 days *in vivo* (*43–45*). In contrast, the minor acetylated adduct, 3-(deoxyguanosin-N^2-yl)-AAF, which accounts for 5% of the binding, is a persistent lesion in hepatic DNA (*44,45*).

The majority of adducts found in rat liver are nonacetylated products that for many years resisted identification. Although they were suspected to be C8-deoxyguanosine derivatives, these adducts tended to undergo decomposition during DNA isolation and hydrolysis. It is now known that this was due to a base-catalyzed opening of the imidazole ring that yielded two pyrimidine adducts (*46*). When the isolation and hydrolysis were performed under milder conditions, chromatographic analysis of rat liver DNA indicated the presence of one major nonacetylated adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene (AF) (*47,48*) (Fig. 8). This has been confirmed by both immunoassays (*50*) and ^{32}P-postlabeling (*51*) techniques. As was observed with 3-(deoxyguanosin-N^2-yl)-AAF, this nonacetylated adduct is relatively persistent in rat liver DNA (*47,49–52*).

Recently Gupta and Dighe (*24*) have examined in greater detail adduct formation and removal in rat liver DNA after a single intraperitoneal injection of *N*-hydroxy-AAF. While their data generally agree with earlier studies, there are a couple of important exceptions. First, due to the sensitivity of their ^{32}P-postlabeling method, they were able to detect many more adducts. Two of these, which accounted for 50% of the initial binding, were originally thought to be a new type of adduct but have subsequently been found to result from incomplete hydrolysis of the modified DNA (Gupta, personal communication). Nevertheless, it is clear from these results, as well as their earlier study (*51*), that a greater variety of adducts than originally suspected can be formed from AAF. Second, when adduct formation was measured as early as 30 min after treatment, there was a substantially greater proportion of acetylated adducts than previously reported.

In principle, a number of metabolic pathways can lead to the adducts detected in rat liver. Through the use of a sulfotransferase inhibitor, pentachlorophenol, Meerman et al. (*48,53*) have provided evidence that the predominant route to acetylated adducts is through the formation of *N*-sulfonyloxy-AAF. The situation regarding the nonacetylated adducts is less clear and evidence has been presented supporting *N*, *O*-acyltransferase-catalyzed formation of *N*-acetoxy-AF (*21,54,55*), direct reaction by *N*-hydroxy-AF (*56*), and AcCoA-dependent formation of *N*-acetoxy-AF(*22*).

Relatively few studies have been performed where hepatic adducts have been examined after multiple or chronic administration of AAF or *N*-hydroxy-AAF. In one experiment, both male and female Sprague-Dawley rats were administered up to four doses of *N*-hydroxy-AAF at biweekly intervals (*49*). *N*-(Deoxyguanosin-8-yl)-AAF was detected in the hepatic DNA from male

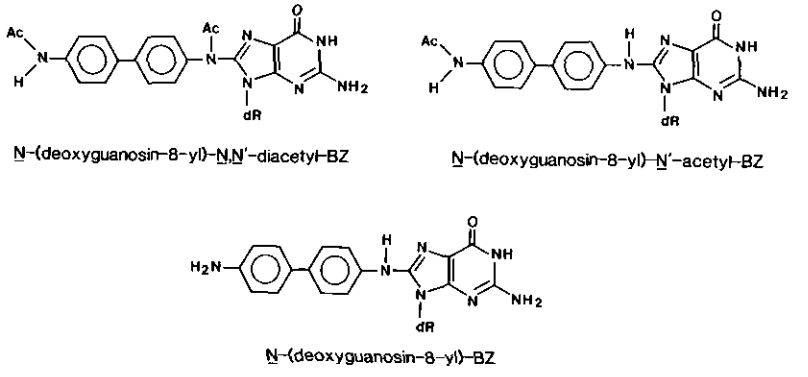

FIGURE 9. DNA adducts derived from benzidine.

rats only and only on the day after dosing, whereas 3-(deoxyguanosin-N^2-yl)-AAF could be found both 1 and 14 days after treatment and increased in an additive manner upon repeated administration of the carcinogen. *N*-(Deoxyguanosin-8-yl)-AF was the major adduct observed in the liver DNA from males and also appeared to increase in an additive manner. Only nonacetylated adducts were detected in the hepatic DNA from females, which is consistent with the low sulfotransferase levels in females of this strain (*57*). The total concentration of adducts was higher in females and these adducts tended to persist. Since females are resistant to *N*-hydroxy-AAF-induced hepatocarcinogenesis (*58*), these results emphasize that adduct persistence alone is not predictive of carcinogenesis.

In a series of experiments, Poirier et al. (*50,59,60*) have used immunoassays to analyze adduct formation and removal during dietary administration of AAF. When rats were given either 0.02 or 0.04% AAF, the concentration of adducts in the liver reached a plateau of about 300 pmole/mg DNA after approximately 3 weeks of feeding. Although acetylated adducts originally made a significant contribution to the total adduct concentration, by the time equilibrium was reached almost all of the adducts were nonacetylated. By using a combination of radioactive and nonradioactive AAF (*60*), they demonstrated that the capability of the liver to form and remove adducts remained constant for at least 1 month and that adduct removal did not depend upon continued carcinogen ingestion. In addition, they found the removal of adducts to be biphasic, which resulted in a significant proportion of the adducts being persistent. From these data a model was proposed in which there were two distinct regions in the genome: one susceptible to fast repair, and another where repair occurred much slower. Although a much greater number of adducts were formed in the fast repair region, the model predicted that the persistent lesions resided exclusively in the slow repair region.

The adducts formed from AAF or *N*-hydroxy-AAF have also been examined in rat kidney and mammary gland. After multiple doses of *N*-hydroxy-AAF or after dietary AAF, binding to kidney DNA was between 20 and 50 % of that found in hepatic DNA (*49,50*). The only adduct detected was *N*-(deoxyguanosin-8-yl)AF and this lesion persisted for at least 1 month following carcinogen administration. *N*-(Deoxyguanosin-8-yl)-AF was also the only adduct found in rat mammary gland epithelium after a single intraperitoneal dose of *N*-hydroxy-AAF (*61*). In this experiment, the maximum adduct concentration was found 4 hr after treatment and then decreased following first-order kinetics with a half-life of 14 days.

Among the aromatic amine carcinogens, AAF is unique in that it induces both bladder and liver tumors in dogs (*41*). In both tissues, *N*-(deoxyguanosin-8-yl)-AF was the major adduct detected after a single dose of AAF (*11*); it was the only adduct in epithelial DNA, whereas other minor adducts were found in hepatic DNA. When the adduct levels were compared at 2 and 7 days following administration, there was an 80% decrease in both tissues which could be due to DNA repair or cytotoxic responses. Since dogs readily deacetylate AAF (*26,27*), the formation of a nonacetylated adduct is consistent with *N*-hydroxy-AF being the reactive intermediate. However, it has recently been reported that dog bladders contain high levels of PHS (*18*) that can activate AF to DNA binding derivatives (*16*). The major adduct formed *in vitro* from PHS-catalyzed cooxidation appears to differ from *N*-(deoxyguanosin-8-yl)-AF (*62*), but its identity is not known. Whether or not this adduct is formed *in vivo* also remains to be established.

When *N*-hydroxy-AAF was administered intraperitoneally to 12-day-old B6C3F_1 mice, a high incidence of hepatic tumors resulted and this could be decreased by prior treatment with the sulfotransferase inhibitor, pentachlorophenol (*63*). In addition, only a low tumor incidence was found when *N*-hydroxy-AAF was administered to a sulfotransferase-deficient strain. These results suggest that *N*-sulfonyloxy-AAF should be the ultimate carcinogenic metabolite; however, when the hepatic adducts were examined, the major product detected was the nonacetylated derivative *N*-(deoxyguanosin-8-yl)-AF, with the acetylated adducts accounting for only 5% of the binding. Since pentachlorophenol decreased both the tumor yield and adduct formation, it appears that in these mice *N*-hydroxy-AAF is deacetylated to *N*-hydroxy-AF that is then converted to the ultimate carcinogen *N*-sulfonyloxy-AF. Support for this interpretation was provided by the observation that *N*-(deoxyguanosin-8-yl)-AF was formed in *in vitro* sulfotransferase assays conducted with mouse liver cytosol, *N*-hydroxy-AF and deoxyguanosine (*63*). Interestingly, when the model ultimate carcinogen *N*-acetoxy-AAF was administered to mice through the orbital sinus, the major adduct detected in lung (target) and liver (nontarget) DNA was *N*-(deoxyguanosin-8-yl)-AF, instead of the acetylated adducts which would have been obtained if this compound was reacted with DNA *in vitro* (Drinkwater and Beland, unpublished observations). The mechanism for this is not known but may involve *N*-deacetylation of *N*-acetoxy-AAF to *N*-acetoxy-AF.

Benzidine

Benzidine (BZ) is a urinary bladder carcinogen in humans (*64–66*) and dogs (*25*), and a hepatocarcinogen in rats, mice, and hamsters (*66–69*). Since BZ has two amino functions, its metabolic activation pathways are potentially more complicated than the monofunctional aromatic amines. In order to elucidate these pathways, BZ and its *N*-acetylated derivatives were administered to rats and the binding to hepatic DNA was compared (*70–72*). The relative order of binding was *N*-acetyl-BZ> BZ >> *N*, *N'*-diacetyl-BZ, which indicated that monoacetylation was part of the activation sequence but that the addition of a second acetyl group resulted in a detoxified product. Based upon this information, *N*-hy-

droxy-*N'*-acetyl-BZ, a suspected activated derivative of *N*-acetyl-BZ was synthesized, reacted with DNA, and the adduct profile was compared to that obtained *in vivo*. Using this approach, the major *in vivo* adduct was identified as *N*-(deoxyguanosin-8-yl)-*N'*-acetyl-BZ (Fig. 9), a product derived either directly from *N*-hydroxy-*N'*-acetyl-BZ or after *O*-esterification of this *N*-hydroxy arylamine. When the extent of binding was compared at 1 and 7 days after a single intraperitoneal injection of *N*-acetyl-BZ, the concentration of this adduct was found to decrease by 60%. Similar kinetics of removal had been observed earlier by Martin and Ekers (*73*), who found a 40% decrease in hepatic DNA binding between 1 and 2 days and then a constant binding level for at least an additional 4 weeks. Subsequently, by using *N*, *N'*-diacetylbenzidine with higher specific activity, a second adduct, *N*-(deoxyguanosin-8-yl)-*N*,*N'*-diacetyl-BZ, was detected in rat liver *in vivo* at approximately 15% of the level of the major adduct (*72*).

The adducts formed from BZ or its *N*-acetylated derivatives have also been examined in other experimental animals. In mice, a single adduct, *N*-(deoxyguanosin-8-yl)-*N'*-acetyl-BZ, was found following administration of BZ in the drinking water for 1 week (*70*). The highest binding was observed immediately after treatment; it decreased by about 50% in 1 day, and then remained at a constant level for at least an additional week. The extent of binding and kinetics of removal of this adduct were the same in male and female mice, although females were much more susceptible to hepatic tumor induction by BZ (*69*). In hamsters (*72*), the same adduct, *N*-(deoxyguanosin-8-yl)-*N'*-acetyl-BZ was found in liver DNA following a single IP injection of *N*-acetyl-BZ. The concentration of this adduct was 50% of that observed in rats, but the kinetics of removal appeared similar with approximately a 50% reduction occurring between 1 and 7 days after treatment.

BZ-derived adducts have also been examined in dogs (*11*). As noted earlier, BZ is a bladder carcinogen in this species (*25*) which may be due to their low *N*-acetylase activity (*26,27*). When a single dose of BZ was given to dogs, a low level of binding could be detected in urothelial DNA (*11*). Although a much higher binding was observed when *N*-acetyl-BZ was given, < 10% appeared to be *N*-(deoxyguanosin-8-yl)-*N'*-acetyl-BZ. Both BZ and *N*-acetyl-BZ gave very high levels of hepatic DNA binding but < 1% could be released upon a standard enzymatic hydrolysis as opposed to the 70 to 90% with other arylamine carcinogens. The identity of these products has not yet been determined.

Since high levels of PHS have been detected in dog bladder (*18*), cooxidation of BZ by this enzyme could result in some of the binding observed *in vivo*. In addition, the liver has a variety of peroxidases that could catalyze the hepatic oxidation of BZ to DNA-binding derivatives. Peroxidase-mediated cooxidation of BZ *in vitro* has been demonstrated (*18,74*), and a major DNA adduct arising from this oxidation has recently been identified as *N*-(deoxyguanosin-8-yl)-BZ (Fig. 9) (*75*). Whether or not this adduct accounts for some of the unidentified adducts in the bladder and liver remains to be determined. The presence of two amine functions in BZ also offers the possibility that dyelike crosslinked products (i.e., DNA–DNA or DNA–protein) may be formed during its oxidation. This would presumably be a minor pathway in rodent species that readily *N*-acetylate BZ.

N,N-Dimethyl-4-aminoazobenzene, *N*-Methyl-4-aminoazobenzene and 4-Aminoazobenzene

N,N-Dimethyl-4-aminoazobenzene (DAB) was among the first carcinogens found that induced tumors at a location removed from the site of application (*76*). This observation, plus DAB's high molar extinction coefficient at visible wavelengths (which facilitated its study in the days preceding radiolabeled compounds), resulted in a number of investigations being conducted with this aminoazo dye (*2*). The first step in the metabolic activation of DAB is a cytochrome P-450-catalyzed oxidative *N*-demethylation to *N*-methyl-4-aminoazobenzene (MAB) (*2,77*). Since a second *N*-demethylation, which yields 4-aminoazobenzene (AB), generally results in a decrease in carcinogenicity, most studies have concentrated on MAB, which is further activated by FMO-catalyzed *N*-oxidation to *N*-hydroxy-MAB and then is converted to an ultimate carcinogen through PAPS-dependent formation of *N*-sulfonyloxy-MAB.

A number of adducts have been characterized from MAB *in vivo*. In rat liver, a target for MAB, two adducts were detected following the administration of a single oral dose. The major adduct, *N*-(deoxyguanosin-8-yl)-MAB (Fig. 10), which was first described by Lin et al. (*78*), initially accounted for 70% of the bound material but was rapidly removed and could not be detected 7 days after treatment (*77–79*). The second adduct was identified as 3-(deoxyguanosin-N^2-yl)-MAB and was found to be a persistent lesion whose concentration remained constant for at least 2 weeks (*77,79*). When multiple doses of MAB were administered and the adducts examined 8 hr after treatment, a third product was detected and subsequently identified as 3-(deoxyadenosin-N^6-yl)-MAB (*80*). Each of the adducts increased in concentration with repeated administration with the rate of increase being faster with 3-(deoxyguanosin-N^2-yl)-MAB and 3-(deoxyadenosin-N^6-yl)-MAB. These kinetics are consistent with the rapid repair of *N*-(deoxyguanosin-8-yl)-MAB in rat liver. More recently, the adduct profile has been determined after feeding MAB to Sprague-Dawley rats for 1,3, or 5 weeks (*81*). Immediately after treatment, the binding to hepatic DNA was 3- to 10-fold higher than that found in the nontarget tissues, kidney and spleen. Within 3 days the hepatic adduct concentration had decreased by 50%; and when the adduct profiles were determined, the only adduct that appeared to persist was 3-(deoxyguanosin-N^2-yl)-MAB.

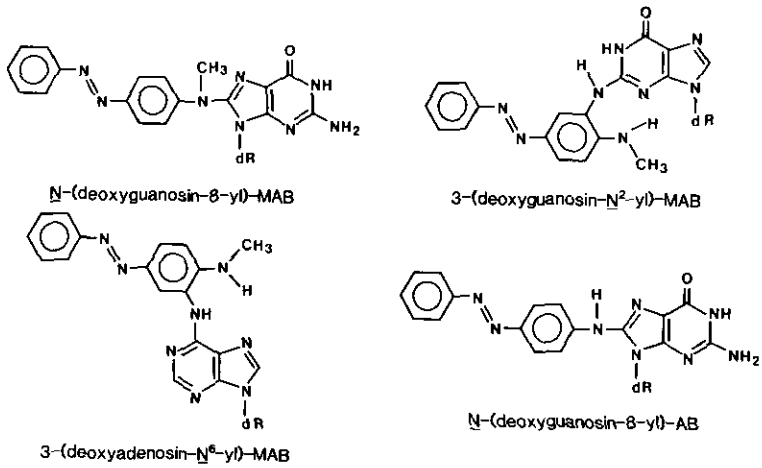

FIGURE 10. DNA adducts derived from *N,N*-dimethyl-4-aminoazobenzene, *N*-methyl-4-aminoazobenzene and 4-aminoazobenzene.

Tarpley et al. (*82*) have examined the hepatic DNA adducts in 12-day-old mice administered a single IP injection of either MAB or DAB. At least 12 products were detected due, in part, to a *cis-trans* isomerism about the azo linkage. As had been observed with rats, the major adduct was *N*-(deoxyguanosin-8-yl)-MAB, which was rapidly removed from the DNA with 70% being lost within 10 days after administration. Significant quantities of 3-(deoxyguanosin-N^2-yl)-MAB were also found, and in a manner similar to what was found in rat liver, this adduct was relatively persistent with only 30% being removed within 10 days.

Although AB is not carcinogenic to adult rats, recent studies indicate that it is nearly as hepatocarcinogenic as MAB and DAB in preweanling mice (*83,84*). When the hepatic DNA adducts were examined 1 day following a single IP injection of AB, only one adduct, *N*-(deoxyguanosin-8-yl)-AB, was detected (*84*). The same adduct was found in DAB-treated mice, although it was only about 25% of the level observed in mice administered AB. In addition, two other adducts were identified in the DAB-treated animals: *N*-(deoxyguanosin-8-yl)-MAB and 3-(deoxyguanosin-N^2-yl)-MAB. In light of the observation that *N*-hydroxy-AF is activated by formation of a sulfate ester in mouse liver and that this tissue contains very low levels of *N,O*-acyltransferase and acetyl CoA-dependent *O*-acetylase, it appears likely that the major pathway leading to hepatic AB adducts is through the formation of *N*-sulfonyloxy-AB.

2-Acetylaminophenanthrene

2-Acetylaminophenanthrene(AAP) is another aromatic amide which gives substantial levels of hepatic DNA binding (*24,85,86*) although it is not hepatocarcinogenic, either with (*86,87*) or without (*88*) promotion. In order to determine the reason for this, Scribner and Koponen (*85*) compared the hepatic DNA adducts formed from AAP to those obtained from AAF following a single IP injection to male Fischer rats. Both compounds gave approximately equal levels of binding and a substantial amount of persistent adducts; however, only nonacetylated adducts, of which two were major and two were minor, were detected from AAP. Equal levels of binding were also detected when rats were fed either of the aromatic amides (*86*). More recently, Gupta and Dighe have reexamined the kinetics of formation and removal of AAP adducts following a single administration of *N*-hydroxy-AAP (*24*). At 4 hr, the hepatic DNA binding of *N*-hydroxy-AAP was much lower (approximately 10%) than that observed with *N*-hydroxy-AAF. However, by 24 hr the binding was nearly equal, and, 10 days after carcinogen administration, the binding detected from *N*-hydroxy-AAP was substantially higher than that found with *N*-hydroxy-AAF. Examination of the adduct profile revealed two major and at least eight minor adducts all of which could be formed by reacting *N*-hydroxy-2-aminophenanthrene (AP) with calf thymus DNA. Thus, in accord with the earlier results of Scribner and Koponen (*85*), all of the adducts appeared to be nonacetylated. Further insight into the identity of the adducts was obtained by reacting *N*-hydroxy-AP with synthetic deoxynucleoside polymers which indicated that the two major adducts were derived from substitution of deoxyguanosine. Preliminary spectroscopic data suggest that one of these products is *N*-(deoxyguanosin-8-yl)-AP (*89*) (Fig. 11), similar to a guanosine product originally reported by Scribner et al. (*86*).

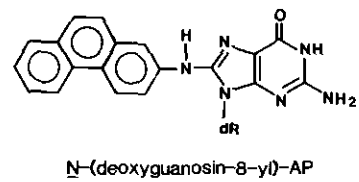

FIGURE 11. DNA adduct derived from 2-acetylaminophenanthrene.

Conclusions

Over the last decade, the identification of aromatic amine-DNA adducts has provided new insights into the mechanisms of metabolic activation, mutagenicity and carcinogenicity. In general, the major sites of carcinogen modification in DNA have been C8 and N^2 of guanine, with substitution at O^6 of guanine, and N^6 and C8 of adenine occurring to a lesser extent. Depending upon the metabolic activation pathway involved, the aromatic amino groups of these adducts have been found to be acetylated, nonacetylated or methylated. In fact, the identity of these adducts, along with the overall adduct profiles observed *in vivo*, has provided strong evidence for the role of specific metabolic conjugation reactions that have been demonstrated *in vitro*.

Although target-tissue specificity of aromatic amine carcinogens has correlated with the levels of DNA modification and with the relative persistence of many of these adducts, several exceptions have been noted in this review. Thus, it is clear that adduct formation *per se* is not sufficient for tumor initiation (fixation of a heritable lesion that can lead to neoplasia) and that other processes, such as error-prone repair and cellular replication, play an essential role. In addition, the relative biological potency of aromatic amine–DNA adducts may differ appreciably from one another as evidenced by the differing mutation efficiencies in *Salmonella typhimurium* TA1538 and TA1535 (*11*), and in Chinese hamster ovary cells (*90*). Furthermore, mutational hotspots have been found in aromatic amine-modified DNA (*91*), which indicates that the biological activity of adducts is further influenced by specific DNA sequences.

Analysis of carcinogen-induced changes in DNA sequence (*91–93*) has often revealed base deletions (frameshifts) which is consistent with *in vitro* studies in which the replication of aromatic amine-modified DNA has resulted in chain termination (*94,95*). In addition, aromatic amine substitution has also led to transversion mutations (*93*). These aromatic amine-induced mutations are thought to arise from conformational changes in DNA, including conversion of the modified base from an *anti* to *syn* conformation (*11,91,94,96*). This, in turn, results in alterations in DNA conformation (B to Z; B/Z junctions; altered B-form) which has been suggested to lead to base deletions and specific mispairings (*94,97–100*). For example, C8 and N^2-substituted guanines have been predicted to result in G to T or G to C transversions (*93,99*), O^6-modified guanines in G to A transitions (*8*), and N^6- and C8-substituted adenines in A to T or A to C transversions (*99*). Therefore, it may not be coincidental that activation of cellular transforming genes of the *ras* family (c-ras^H, c-ras^K, and c-ras^N), which has been implicated in bladder, colon, liver, mammary, lung, nervous system and embryonal tumors, can be achieved by G to T transversions (*101–103*) and G to A transitions (*104,105*) at coding position 12 and A to T transversions (*106*) at coding position 61 of the first exon. Additional studies of the consequences of aromatic amine–DNA adducts should be pursued and may provide important data on the molecular biology of cancer.

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